NOVEL PLANT PROMOTERS AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of, and hereby incorporates by reference, U.S. non-provisional application 09/766,399, filed January 19, 2001, and U.S. provisional patent application 60/177,437, filed January 21, 2000.

FIELD OF THE INVENTION

The present invention relates to the field of plant molecular biology, more particularly to regulation of gene expression in plants.

BACKGROUND OF THE INVENTION

Expression of heterologous DNA sequences in a plant host is dependent upon the presence of an operably linked promoter that is functional within the plant host. Choice of the promoter sequence will determine when and where within the organism the heterologous DNA sequence is expressed. Thus, where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized. In contrast, where gene expression in response to a stimulus is desired, inducible promoters are the regulatory element of choice. Where expression in particular organs is desired, tissue specific promoters are utilized. Additional regulatory sequences upstream and/or downstream from the core promoter sequence may be included in expression constructs of transformation vectors to bring about varying levels of constitutive or inducible expression of heterologous nucleotide sequences in a transgenic plant.

Frequently it is desirable to have constitutive expression of a DNA sequence throughout the cells of an organism. For example, increased resistance of a plant to infection by soil- and air-borne pathogens might be accomplished by genetic manipulation of the plant's genome to comprise a constitutive promoter operably linked to a heterologous pathogen-resistance gene such that pathogen-resistance proteins are continuously expressed throughout the plant's tissues.

Alternatively, it might be desirable to inhibit expression of a native DNA sequence within a plant's tissues to achieve a desired phenotype. In this case, such inhibition might be accomplished with transformation of the plant to comprise a

constitutive promoter operably linked to an antisense nucleotide sequence, such that constitutive expression of the antisense sequence produces an RNA transcript that interferes with translation of the mRNA of the native DNA sequence.

Thus, isolation and characterization of promoters and promoter elements that can serve as regulatory regions for expression of heterologous nucleotide sequences of interest are needed for genetic manipulation of plants.

SUMMARY OF THE INVENTION

Compositions and methods for regulating expression of heterologous nucleotide sequences in a plant are provided. The compositions comprise novel nucleotide sequences for synthetic multimeric promoter element regions (SMPERs) and plant promoters comprising the SMPERs. Particularly, plant promoters comprising one or more SMPERs that enhance expression directed by the promoter are provided.

Methods for expressing a heterologous nucleotide sequence in a plant using the promoter sequences disclosed herein are provided. The methods comprise transforming a plant cell with a transformation vector that comprises a heterologous nucleotide sequence operably linked to one of the plant promoters of the present invention and regenerating a stably transformed plant from the transformed plant cell. In this manner, expression levels in a plant cell, plant organ, plant tissue or plant seed can be controlled.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequences of 64 defined or putative promoter elements or transcription factor binding sites. Promoter elements selected for synthesis of SMPERs are designated by an asterisk.

Figure 2 shows the 2-dimensional (8X8) register for pooling of transcription factor binding sites and/or promoter elements Em1a (SEQ ID NO.: 1), ABRE1 (SEQ ID NO.: 2), ABRE A (SEQ ID NO.: 3), Prolamin P-box (SEQ ID NO.: 4), Z2 and Z3 box (SEQ ID NO.: 5), 35S AS-2 (SEQ ID NO.: 6), 35S AS-1 (SEQ ID NO.: 7), OCS ele (SEQ ID NO.: 8), GCC-box (SEQ ID NO.: 9), GH3 D1 (SEQ ID NO.: 10), GH3 D3 (SEQ ID NO.: 11), P3 (SEQ ID NO.: 12), GT-1 rbcS3A (SEQ ID NO.: 13), TCA motif (SEQ ID NO.: 14), C-repeat/DRE (SEQ ID NO.: 15), HSE (SEQ ID NO.: 16), ERE (SEQ ID NO.: 17), gln2 PR box (SEQ ID NO.: 18), HBP-1a (SEQ ID NO.: 19), A1

PROMOTER (SEQ ID NO.: 20), Bz1 PROMOTER (SEQ ID NO.: 21), CHS promoter (SEQ ID NO.: 22), BoxII (SEQ ID NO.: 23), phyA GT-2 (SEQ ID NO.: 24), GT-2 like (SEQ ID NO.: 25), Phy PF1 (SEQ ID NO.: 26), AT-com (SEQ ID NO.: 27), AG site (SEQ ID NO.: 28), AP3 site (SEQ ID NO.: 29), TGAC motif (SEQ ID NO.: 30), CAGT motif (SEQ ID NO.: 31), Dof1/Dof2 (SEQ ID NO.: 32), pr2 oligomer II (SEQ ID NO.: 33), CE1 (SEQ ID NO.: 34), H-box1 (SEQ ID NO.: 35), H-box2 (SEQ ID NO.: 36), lox1 (SEQ ID NO.: 37), PR-2d (SEQ ID NO.: 38), ROL6 (SEQ ID NO.: 39), SGB box 2/3 (SEQ ID NO.: 40), SGB box 6-8 (SEQ ID NO.: 41), MS-BS7 box 1-3 (SEQ ID NO.: 42), MS-BS7 box 22-24 (SEQ ID NO.: 43), AuxRE DR5 (SEQ ID NO.: 44), PCNA IIA (SEQ ID NO.: 45), PAL1 Box E (SEQ ID NO.: 46), myb26 (SEQ ID NO.: 47), GARE (SEQ ID NO.: 48), E8 (SEQ ID NO.: 49), E1RE (SEQ ID NO.: 50), CA (SEQ ID NO.: 51), napA (SEQ ID NO.: 52), HaG3-A-75 (SEQ ID NO.: 53), HaG3-A-111 (SEQ ID NO.: 54), Prolamin box (SEQ ID NO.: 55), TGAC-like (SEQ ID NO.: 56), SP20+6 (SEQ ID NO.: 57), MSA RT1 (SEQ ID NO.: 58), DRE rd29A1 (SEQ ID NO.: 59), DRE rd29A2 (SEQ ID NO.: 60), CGF-1 (SEQ ID NO.: 61), ltp1 D1 (SEQ ID NO.: 62), ENBP1 (SEQ ID NO.: 63), and MRE (SEQ ID NO.: 64).

Figure 3 depicts promoter elements characterized as having strong binding to maize nuclear extracts. These promoter elements are shaded.

Figure 4 is a schematic representation of the expression cassette in the AdhI intron plus expression vector, comprising specific synthetic multimeric promoter element regions. The A designations (e.g. A18) refer to clone numbers comprising the particular SMPER depicted. Arrows indicate the orientation of each promoter element. Identity of each promoter element is shown by the key at the bottom. LexA depicts the negative control. " +++" indicates high enhancer activity.

Figure 5 is a schematic representation of the expression cassette in the AdhI intron minus expression vector, comprising specific synthetic multimeric promoter element regions. The A designations (e.g. A42) refer to clone numbers comprising the particular SMPER depicted. Arrows indicate the orientation of each promoter element. Identity of each promoter element is shown by the key at the bottom. LexA depicts the negative control. " +++" indicates high enhancer activity.

Figures 6a, 6b, 6c, and 6d depict results of transient assays for luciferase activity in extracts of maize seedlings transformed with the indicated SMPER constructs.

Figures 7-14 provide the respective nucleotide sequences for SMPER A15 (SEQ ID NO. 65), A18 (SEQ ID NO. 66), A23 (SEQ ID NO. 67), A24 (SEQ ID NO. 68), A42 (SEQ ID NO. 69), A44 (SEQ ID NO. 70), A48 (SEQ ID NO. 71), and A51 (SEQ ID NO. 72), respectively. Spacer sequences are designated by underscoring. Individual promoter elements are designated according to the corresponding element names shown in Figure 1.

DETAILED DESCRIPTION OF THE INVENTION

Compositions of the present invention are directed to novel nucleotide sequences for synthetic multimeric promoter element regions (SMPERs) and plant promoters comprising the SMPERs. Particularly, plant promoters are provided comprising at least one SMPER that enhances transcription directed by the promoter. The SMPERs comprise novel arrangements of individual promoter elements. See, for example, Figure 1. In particular, specific combinations comprising the promoter elements PCNA IIA, GT-2, ABRE 1, As-1 and DRE 1 are provided.

The multimeric promoter element regions of the invention, and the plant promoters of the invention comprising the multimeric promoter element regions, are synthetic. By "synthetic" is intended that nucleotide sequences of the multimeric promoter element regions of the invention, or that of the plant promoters of the invention comprising the multimeric promoter element regions, are not found in nature.

By "synthetic multimeric promoter element region" or "SMPER" is intended a nucleic acid having a nucleotide sequence comprising more than one promoter element, wherein the arrangement of the multimeric combination of the promoter elements is not found in nature. That is, the invention recognizes that the promoter elements can be provided in any sequence or arrangement to provide a SMPER. Such SMPER is then tested for its effect on transcription. It is recognized that the elements may be presented in any order. In some instances, elements may be duplicated, i.e., more than one copy of an individual element may be present in the SMPER. Using the methods described herein, combinations of promoter elements can be tested for their effect on transcription. In this manner, any combination is

encompassed by the invention. Preferred SMPERs of the invention comprise promoter elements including but not limited to PCNA IIA, GT-2, ABRE 1, As-1 and DRE1.

The SMPERs of the invention may be used with any promoter, native or synthetic. More particularly, the SMPERs may be used with any plant promoter, native or synthetic. By "plant promoter" is intended a promoter capable of driving expression in a plant cell.

In reference to a promoter, by "native" is intended a promoter capable of driving expression in a particular cell, wherein the nucleotide sequence of the promoter is found in that cell in nature. That is, the nucleotide sequence of a native promoter can be isolated from the cell, or the corresponding cell source, without introduction of the promoter to the cell, the cell source, or an ancestor thereof. By "cell source" is intended an organism or tissue from which the cell is derived.

In reference to a promoter, by "synthetic" is intended a promoter capable of driving expression in a particular cell, wherein the nucleotide sequence of the promoter is not found in nature. That is, the nucleotide sequence of a synthetic promoter cannot be isolated from the cell, or the corresponding cell source, without having introduced the promoter to the cell, the cell source, or an ancestor thereof. Thus, the combination of the promoters and the SMPERs are synthetic. These combinations are not found in nature and cannot be isolated from a native plant, plant cell, or plant tissue.

The individual promoter elements PCNA IIA, GT-2, ABRE1, As-1 and DRE1 are described in Figure 1. The methods for synthesizing and isolating the plant promoters of the invention are provided in the Examples described below.

The nucleotide sequences of SMPERs of the present invention comprising specific combinations of promoter elements PCNA IIA, GT-2, ABRE1, As-1 and DRE1 are set forth in Figures 7-14 (SEQ ID NOS: 65-72).

The invention encompasses isolated or substantially purified nucleic acid compositions. An "isolated" or "purified" nucleic acid molecule, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "plant" includes reference to whole plants and their progeny; plant cells; plant parts or organs, such as embryos, pollen, ovules, seeds, flowers, kernels, ears, cobs, leaves, husks, stalks, stems, roots, root tips, anthers, silk

and the like. Plant cell, as used herein, further includes, without limitation, cells obtained from or found in: seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plant cells can also be understood to include modified cells, such as protoplasts, obtained from the aforementioned tissues. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. A particularly preferred plant is *Zea mays*.

By "promoter" or "transcriptional initiation region" is intended a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, and referred to as promoter elements which influence the sequences for the promoter regions disclosed herein. Promoter elements located upstream or 5' to the TATA box are also referred to as upstream promoter elements. In particular embodiments of the invention, the SMPERs of the invention are positioned upstream or 5' to the TATA box. However, the invention also encompasses plant promoter configurations in which the SMPERs are positioned downstream or 3' to the TATA box.

The promoter elements of the invention may act as enhancers or suppressors of expression.

Enhancers are nucleotide sequences that act to enhance or increase the expression directed by a promoter region. An enhancer can be identified by comparing the expression level directed by a sample promoter comprising the enhancer sequence to be tested placed at any position upstream or downstream of the promoter, relative to a control promoter that does not comprise the sequence in question. Known individual enhancer elements for plants include, for example, the SV40 enhancer region, the 35S enhancer element, and the like. The SMPERs of the invention enhance expression of coding sequences operably linked to the plant promoters comprising the SMPERs. Accordingly, the SMPERs of the invention may act as enhancers. Li et al. (1999), Nature Biotechnology: 17: 241-245, describe random assembly of muscle promoter elements for achieving enhanced promoter activity in muscle.

By "suppressors" are intended nucleotide sequences that mediate suppression or decrease in the expression directed by a promoter region. That is, suppressors

are the DNA sites through which transcription repressor proteins exert their effects. Suppressors can mediate suppression of expression by overlapping transcription start sites or transcription activator sites, or they can mediate suppression from distinct locations with respect to these sites. The SMPERs of the invention may act as suppressors.

The SMPERs may be operably linked to any promoter of interest. While not a limitation, it may be preferable to use core promoters. By "core promoter" is intended a promoter without regulatory promoter elements such as enhancers, suppressors, and the like. Promoters of interest include but are not limited to constitutive, weak, pathogen-indicible, wound-inducible, chemical-regulated, and tissue-specific promoters, including but not limited to leaf-specific, root-specific, and seed-specific promoters.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 (U.S. Patent No. 6,072,050); the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. patent 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

See also the copending application entitled "Constitutive Maize Promoters", U.S. Application Serial No. 09/257,584, filed February 25, 1999, and herein incorporated by reference.

Such pathogen-inducible promoters include but are not limited to those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol. 89*:245-254; Uknes *et al.* (1992) *Plant Cell 4*:645-656; and Van Loon (1985) *Plant Mol. Virol. 4*:111-116. See also the copending application entitled "Inducible Maize Promoters", U.S. Application Serial No. 09/257,583, filed February 25, 1999, herein incorporated by reference. Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol. 9*:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions 2*:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.*

2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA 93*:14972-14977. See also, Chen *et al.* (1996) *Plant J. 10*:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2507-2511; Warner *et al.* (1993) *Plant J. 3*:191-201; Siebertz *et al.* (1989) *Plant Cell 1*:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path. 41*:189-200).

Such wound-inducible promoters include but are not limited to potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath. 28*:425-449; Duan *et al.* (1996) *Nature Biotechnology 14*:494-498); wun1 and wun2, US Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet. 215*:200-208); systemin (McGurl *et al.* (1992) *Science 225*:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol. 22*:783-792; Eckelkamp *et al.* (1993) *FEBS Letters 323*:73-76); MPI gene (Corderok *et al.* (1994) *Plant J. 6(2)*:141-150); and the like, herein incorporated by reference.

Such chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Such tissue-preferred promoters include but are not limited toYamamoto *et al.* (1997) *Plant J. 12(2)*255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol. Gen Genet. 254(3)*:337-343; Russell *et al.* (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci.*

USA 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-specific promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kwon *et al.* (1994) *Plant Physiol. 105*:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Gotor *et al.* (1993) *Plant J. 3*:509-18; Orozco *et al.* (1993) *Plant Mol. Biol. 23(6)*:1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590.

Root-specific promoters are known and can be selected from the many available in the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol. 20(2)*: 207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell 3(10)*:1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol. 14(3)*:433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell 3(1)*:11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell 2(7)*:633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) *79(1)*:69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J. 8(2)*:343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol. 29(4)*:759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.*

25(4):681-691). See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays 10*:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase); and celA (cellulose synthase) (see the copending application entitled "Seed-Preferred Promoters," U.S. Application Serial No. 09/377,648, filed August 19, 1999, herein incorporated by reference). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

Generally, the plant promoter sequences of the present invention, when operably linked to a heterologous nucleotide sequence of interest and inserted into a transformation vector, control constitutive expression of the heterologous nucleotide sequence in the cells of a plant stably transformed with this vector. By "constitutive" is intended expression in the cells throughout a plant at most times and in most tissues. It is recognized that depending on the particular host plant or tissue, the particular SMPER or promoter comprising the SMPERs, and variants and fragments thereof, could be used to control tissue-preferred or tissue-specific expression.

By "heterologous nucleotide sequence" is intended a sequence that is not naturally occurring with the promoter sequence. The SMPERs and the plant promoters of the invention comprising the SMPERs are not found in nature. Therefore, any sequence of interest operably linked to a promoter comprising the SMPERs of the invention is a heterologous nucleotide sequence. While this linked nucleotide sequence is heterologous to the promoter sequence, it may be homologous (native) or heterologous (foreign) to the plant host.

The isolated SMPER sequences of the present invention, and plant promoter sequences comprising the SMPERs, can be modified to provide for a range of expression levels of the heterologous nucleotide sequence. Thus, less than the entire promoter regions may be utilized and the ability to drive expression of the

coding sequence retained. However, it is recognized that expression levels of the mRNA may be decreased with deletions of portions of the promoter sequences. Likewise, the general nature of expression may be changed.

Modifications of the SMPER sequences of the present invention and of plant promoter sequences comprising the SMPERs can provide for a range of expression. Thus, they may be modified to be weak promoters or strong promoters. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended at levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

The nucleotide sequences for the plant promoters of the present invention may comprise the sequences set forth in Figures 7-14 (SEQ ID NO: 65-72) or any sequence having substantial identity to the sequences. By "substantial identity" is intended a sequence exhibiting substantial functional and structural equivalence with the sequence set forth. Any functional or structural differences between substantially identical sequences do not affect the ability of the sequence to function as a promoter as disclosed in the present invention. Thus, the plant promoter of the present invention will direct enhanced expression of an operably linked heterologous nucleotide sequence. Two SMPER nucleotide sequences are considered substantially identical when they have at least about 80%, preferably at least about 85%, more preferably at least about 90%, still more preferably at least about 95%, and most preferably at least about 98% sequence identity.

Fragments and variants of the SMPER nucleotide sequences set forth herein are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence that is longer than the shortest individual promoter element contained in the particular portion. Fragments of a nucleotide sequence may retain biological activity and hence enhance expression of a nucleotide sequence operably linked to a synthetic promoter comprising the SMPER. (*See* Lam *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:7890; *See also* Oliphant *et al* (1989) *Mol. Cell Biol. 9*: 2944-2949; Niu and Guiltinan (1994) *Nucleic Acid Res. 22*: 4969-497; Oeda, et al EMBO J. 10:1793; and Catron *et al.* (1993) *Mol. Cell Biol. 13*: 2354-2365.) Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes or PCR primers generally do not retain biological activity. Thus, fragments of a nucleotide sequence may range from at least 7 to 10, or about 21, 25, 28, or 29

nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full length of a nucleotide sequence of the invention.

A biologically active portion of a promoter comprising the SMPERs of the invention can be prepared by synthesizing a portion of one of the promoter nucleotide sequences and assessing the activity of the fragment. Nucleic acid molecules that are fragments of a promoter nucleotide sequence comprise at least 21, 50, 75, 100, 150, or 200 nucleotides, or up to the number of nucleotides present in a full-length promoter nucleotide sequence disclosed herein (for example, 413, 392, 314, 278, 348, 198, 302, or 157 nucleotides for Figures 7, 8, 9, 10, 11, 12, 13, or 14 (SEQ ID NO: 65-72), respectively).

Variants of these promoter fragments, such as those resulting from site-directed mutagenesis, are encompassed by the compositions of the present invention.

The invention encompasses variants of the SMPERs and of the plant promoter sequences comprising the SMPERs. By "variants" is intended substantially identical sequences. Naturally occurring variants of the individual promoter element sequences can be identified and/or isolated with the use of well-known molecular biology techniques, as, for example, with PCR and hybridization techniques as outlined below. The invention encompasses variants of the SMPERs and plant promoter sequences disclosed herein in which one or more of the individual promoter elements are substituted by a natural variant of that element. For example, and without limitation, the element ABRE 1 could be substituted by the ABRE A; and/or DRE1 could be substituted by DRE2.

The invention encompasses variants of the SMPERs and plant promoter sequences disclosed herein in which one or more of the individual promoter elements is in the alternative orientation. By "orientation" is intended the 5' to 3' (sense) or the 3' to 5' (antisense) configuration of a promoter element sequence contained in a contiguous strand, relative to the configuration of other promoter elements and/or the TATA box contained in that strand.

The invention encompasses plant promoter and SMPER sequences in which the individual promoter elements are separated and/or flanked by spacer sequences. By "spacer sequence" is intended the nucleotide sequence contained in an SMPER that is not a promoter element. The invention also encompasses variants of the SMPERs and plant promoter sequences comprising contiguous multimers of individual promoter elements thereby containing no spacer sequences; variants in

which one or more individual elements are separated or flanked by spacer sequences, and variants comprising spacer sequences that are different than the spacer sequences disclosed herein.

Variant SMPER and promoter nucleotide sequences include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis, but which still exhibit promoter activity. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Generally, a nucleotide sequence of the invention will have at least 80%, preferably 85%, 90%, 95%, up to 98% or more sequence identity to its respective reference promoter nucleotide sequence, and enhance or promote expression of heterologous coding sequences in plants or plant cells.

Variant promoter nucleotide sequences also encompass sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different promoter sequences can be manipulated to create a new promoter possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

Biologically active variants of the promoter sequences should retain promoter activity and thus promote or enhance expression of an operably linked heterologous nucleotide sequence. Promoter activity may be measured by Northern blot analysis. See, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York), herein incorporated by reference. Protein expression indicative of promoter activity can be measured by determining the activity of a protein encoded by the coding sequence operably linked to the particular promoter, including but not limited to such examples

as GUS (b-glucoronidase; Jefferson (1987) *Plant Mol. Biol. Rep.* 5:387), GFP (green florescence protein; Chalfie *et al.* (1994) *Science* 263:802), luciferase (Riggs *et al.* (1987) *Nucleic Acids Res.15(19)*:8115 and Luehrsen *et al.* (1992) *Methods Enszymol. 216*:397-414), and the maize genes encoding for anthocyanin production (Ludwig *et al.* (1990) *Science* 247:449).

It is recognized that the SMPERs are not found in nature. That is, the combination of the individual promoter elements is novel. However, it is also recognized that the nucleotide sequences of the invention may be used to isolate substantially identical sequence fragments from natural sources, particularly plants. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Such methods are generally known in the art and are disclosed in, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Sequences isolated based on their sequence identity to a fragment of the sequences set forth herein are encompassed by the present invention. Such individual elements can be used in a SMPER.

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of identity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g.,

greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in*

Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

In general, sequences that have promoter or enhancer activity and hybridize to the sequences disclosed herein will be at least 80%, 85%, 90%, 95% to 98% or more identical with the disclosed sequences.

Methods of alignment to determine the extent of identity of two sequences are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; the homology alignment algorithm of Needleman *and Wunsch* (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson *and Lipman* (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872*264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG® programs (Accelrys, Inc., San Diego, CA)); and Sequencher (GeneCodes, Ann Arbor, MI). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12,

to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. Alignment may also be performed manually by inspection. For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the sequences disclosed herein is preferably made using GAP (GCG Version 10) with its default parameters, or any equivalent sequence comparison program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having substantially identical nucleotide or amino acid residue matches and a substantially identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 80% sequence identity, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 98%, compared to a sequence of the invention using one of the alignment programs described above using standard or default parameters.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein.

The nucleotide sequences for the SMPERs and promoters of the present invention, as well as variants and fragments thereof, are useful in the genetic manipulation of any plant when operably linked with a heterologous nucleotide sequence whose expression is to be controlled to achieve a desired phenotypic response. By "operably linked" is intended that the transcription or translation of the heterologous nucleotide sequence is under the influence of the promoter sequence. In this manner, the nucleotide sequences for the promoters of the invention are provided in expression cassettes along with nucleotide sequences of interest for expression in the plant of interest.

Such DNA constructs or expression cassettes will comprise a transcriptional initiation region comprising one of the promoter nucleotide sequences of the present invention, or variants or fragments thereof, operably linked to the heterologous nucleotide sequence whose expression is to be controlled by the promoters disclosed herein. Such an expression cassette is provided with a plurality of restriction sites for insertion of the nucleotide sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a heterologous nucleotide sequence of interest, and a transcriptional and translational termination region functional in plant cells. The termination region may be native with the transcriptional initiation region comprising one of the promoter nucleotide sequences of the present invention, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* 1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

The expression cassette comprising the promoter sequence of the present invention operably linked to a heterologous nucleotide sequence may also contain at least one additional nucleotide sequence for a gene to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

Where appropriate, the heterologous nucleotide sequence whose expression is to be under the control of the promoter sequence of the present invention and any additional nucleotide sequence(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred nucleotide sequences. See, for example, U.S. Patent Nos. 5,380,831 and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the heterologous nucleotide sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986)); MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) (Macejak and Sarnow (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling and Gehrke (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965-968. Other methods known to enhance translation and/or mRNA stability can also be utilized, for example, introns, and the like.

In those instances where it is desirable to have the expressed product of the heterologous nucleotide sequence directed to a particular organelle, such as the chloroplast or mitochondrion, or secreted at the cell's surface or extracellularly, the expression cassette may further comprise a coding sequence for a transit peptide.

Such transit peptides are well known in the art and include, but are not limited to, the transit peptide for the acyl carrier protein, the small subunit of RUBISCO, plant EPSP synthase, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments, or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, for example, transitions and transversions, may be involved.

The promoters may be used to drive reporter genes or selectable marker genes. Examples of suitable reporter genes known in the art can be found in, for example, Jefferson *et al.* (1991) in *Plant Molecular Biology Manual,* ed. Gelvin *et al.* (Kluwer Academic Publishers), pp. 1-33; DeWet *et al.* (1987) *Mol. Cell. Biol.* 7:725-737; Goff *et al.* (1990) *EMBO J.* 9:2517-2522; and Kain *et al.* (1995) *BioTechniques* 19:650-655; and Chiu *et al.* (1996) *Current Biology* 6:325-330.

Selectable marker genes for selection of transformed cells or tissues can include genes that confer antibiotic resistance or resistance to herbicides. Examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to chloramphenicol (Herrera Estrella *et al.* (1983) *EMBO J.* 2:987-992); methotrexate (Herrera Estrella *et al.* (1983) *Nature* 303:209-213; Meijer *et al.* (1991) *Plant Mol. Biol.* 16:807-820); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol.* 5:103-108; Zhijian *et al.* (1995) *Plant Science* 108:219-227); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet.* 210:86-91); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res.* 5:131-137); bleomycin (Hille *et al.* (1990) *Plant Mol. Biol.* 7:171-176); sufonamide (Guerineau *et al.* (1990) *Plant Mol. Biol.* 15:127-136); bromoxynil (Stalker *et al.* (1988) *Science* 242:419-423); glyphosate (Shaw *et al.* (1986) *Science* 233:478-481); phosphinothricin (DeBlock *et al.* (1987) *EMBO J.* 6:2513-2518).

Other genes that could serve utility in the recovery of transgenic events but might not be required in the final product would include, but are not limited to, such examples as GUS (b-glucoronidase; Jefferson (1987) *Plant Mol. Biol. Rep.* 5:387), GFP (green florescence protein; Chalfie *et al.* (1994) *Science* 263:802), luciferase (Riggs *et al.* (1987) *Nucleic Acids Res.15(19):*8115 and Luehrsen *et al.* (1992)

Methods Enszymol. 216:397-414), and the maize genes encoding for anthocyanin production (Ludwig *et al.* (1990) *Science* 247:449).

The expression cassette comprising the particular promoter sequence of the present invention operably linked to a heterologous nucleotide sequence of interest can be used to transform any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Pat No. 5,563,055), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated

transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested, to ensure expression of the desired phenotypic characteristic has been achieved.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats (*Avena sativa*), barley (*Hordeum vulgare*), vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus*

rosasanensis), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Plants of particular interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

The promoter sequences and methods disclosed herein are useful in regulating expression of any heterologous nucleotide sequence in a host plant. Thus, the heterologous nucleotide sequence operably linked to the promoters disclosed herein may be a structural gene encoding a protein of interest. Examples of such heterologous genes include, but are not limited to, genes encoding proteins conferring resistance to abiotic stress, such as drought, temperature, salinity, and toxins such as pesticides and herbicides, or to biotic stress, such as attacks by fungi, viruses, bacteria, insects, and nematodes, and development of diseases associated with these organisms.

Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increases, the choice of genes for transformation will change accordingly. General categories of genes of interest

include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like.

Agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch. Hordothionin protein modifications are described in U.S. Patent Nos. 5,990,389, 5,885,801, and 5,885,802, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Patent No. 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson *et al.* (1987) *Eur. J. Biochem. 165*:99-106, the disclosures of which are herein incorporated by reference.

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. Application Serial No. 08/740,682, filed November 1, 1996, and PCT Publication No. WO98/20133, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley *et al.* (1989) *Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs*, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen *et al.* (1986) *J. Biol. Chem. 261*:6279; Kirihara *et al.* (1988) *Gene 71*:359; both of which are herein incorporated by reference); and rice (Musumura *et al.* (1989) *Plant Mol. Biol. 12*:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene 48*:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol. 24*:825); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Patent No. 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science 266*:789; Martin *et al.* (1993) *Science 262*:1432; and Mindrinos *et al.* (1994) *Cell 78*:1089); and the like.

Herbicide resistance traits may include genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in U.S. Patent No. 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

Commercial traits can also be encoded on a gene or genes that could increase, for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxybutyrate synthase), and acetoacetyl-CoA reductase (see Schubert *et al.* (1988) *J. Bacteriol. 170*:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs).

Exogenous products include plant enzymes and products as well as those from other sources including procaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the

nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

Alternatively, the heterologous nucleotide sequence operably linked to one of the promoters disclosed herein may be an antisense sequence for a targeted gene. Thus, sequences can be constructed which are complementary to, and will hybridize with, the messenger RNA (mRNA) of the targeted gene. Modifications of the antisense sequences may be made, as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence similarity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used. When delivered into a plant cell, expression of the antisense DNA sequence prevents normal expression of the DNA nucleotide sequence for the targeted gene. In this manner, production of the native protein encoded by the targeted gene is inhibited to achieve a desired phenotypic response. Thus the promoter is linked to antisense DNA sequences to reduce or inhibit expression of a native protein in the plant.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1: Collection and Identification of Promoter Elements

Sequences of 64 defined or putative promoter elements or transcription factor binding sites were collected, each element 20-40 base pairs(bp) long.
The sequences are shown in Figure 1 in the 5' to 3' (sense) direction. Oligonucleotides (oligos) corresponding to the top (sense) strands and bottom (antisense) strands of these promoter element sequences were synthesized by automated DNA synthesizer. For DNA synthesis, the spacer sequence TAGC was added to all the top strand oligos and GCTA to all the bottom strand oligos to facilitate subsequent DNA manipulation.

The 64 pairs of corresponding sense and antisense synthesized oligos were annealed in individual reactions (88°C for 2 minutes (min.), 65°C for 15 min., 37°C for 15 min., 25°C for 5 min.). Thereafter, the oligos were arranged and registered in an

8x8 format in a microtiter plate. These oligos were pooled using a 2-dimensional pooling strategy (8 horizontal and 8 vertical pools). Each pool contains 8 oligo pairs, as indicated in Figure 2. The 16 pools of oligos were labeled with Klenow enzyme in the presence of P-32-dCTP in separate reactions (200 ng DNA in each 20 µl reaction). The labeled DNA was purified by a spin column (Bio-Gel P-6 spin column, Biorad). These DNA probes were used in DNA-binding reactions with maize nuclear extracts.

Nuclear extracts were prepared using a protocol modified from Green *et al.* (1988) "In vitro DNA Footprinting," in *Plant Molecular Biology Manual,* ed. Gelvin, Schilperoort, and Verma (Kluwer Academic Publishers, Dordrecht) *B11*: 1-22. Seeds were germinated in the dark at 24°C. Roots from 4-day seedlings were collected and 4X volume of the Homogenizing Buffer (25 mM Hepes/KOH pH 7.6, 10 mM MgCl2, 0.3 M sucrose, 0.5% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF) was added. Tissues were dissected into small pieces using a commercial Waring blender at low speed for 10 sec and ground to paste with mortar and pestle. Homogenized tissues were filtered through two layers of miracloth (CalBiochem) and one layer of 70 µm nylon screen. The extracts were centrifuged in a Sorval GSA rotor, 4500 rpm, 15 minutes. Nuclei pellets were then resuspended gently with a paint brush in Homogenizing Buffer and centrifuged as above. This step was repeated once. After the last centrifugation, nuclei were resuspended in Nuclear Lysis Buffer (15 mM Hepes/KOH pH 7.6, 110 mM KCl, 5 mM MgCl2, 1mM DTT, 1 mM PMSF, 5µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A). NaCl was added in a dropwise manner to a final concentration of 0.5 M. Nuclear proteins were extracted from the nuclei by incubation of the NaCl mixture on ice for 40 minutes with gentle shaking. The extract was centrifuged in Sorval SS34 rotor, 16K rpm, for 30 minutes. Supernatants were frozen in liquid nitrogen and stored at –80°C. To continue nuclear extract preparation, frozen nuclear extracts were thawed on ice and ammonium sulfate was added slowly to nuclear extracts to a final concentration of 0.35 mg/ml while stirring. Precipitated nuclear proteins were centrifuged in Sorval SS34 rotor at 16k rpm for 30 min. The pellets were resuspended in Nuclear Extract Buffer (25 mM Hepes/KOH pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol) with 1 mM PMSF, 5µg/ml antipain, 5µg/ml leupeptin and 5µg/ml aprotinin and dialyzed for 6 hours against NEB with 0.1 mM PMSF. The dialyzed nuclear extracts were aliquoted and stored at –80°C until use.

Gel Shift Assays

For DNA-binding reactions, about 1-2 µg aliquots of the nuclear extracts were incubated with the labeled DNA probes (10 ng) in presence of 1 µg poly(dI-dC). The binding reactions were incubated on ice for 5-20 minutes and run on 4% polyacrylamide-0.5 x TBE gel at room temperature for 2 hours. Each of the 16 lanes of the gel corresponded to one pool of oligonucleotides as indicated in Figure 2. The gel was then dried and exposed to Kodak film. Gel shift results indicated that some oligo probes were bound very strongly by factors in maize nuclear extracts, as evidenced by their reduced mobility in the gel. Cross-reference to these strong binding activities from the two dimensional 8x8 pooling register (Figure 2) indicated that these strong binding activities were contributed by the promoter elements PCNA IIA, GT-2, ABRE1, As-1, and DRE1, as indicated in Figure 3.

EXAMPLE 2: Multimerization of Promoter Elements

Because promoter elements PCNA IIA, GT-2, ABRE1, As-1, and DRE1 were bound strongly by factors in maize nuclear extracts, the conclusion was that the transcription factors interacting with the elements are expressed abundantly in maize. Accordingly, the five promoter elements were selected to be synthetically combined into highly active synthetic promoters.

To synthesize the promoter element multimers, the oligos described above (top and bottom strands having spacer sequences) for the five promoter elements were phosphorylated by T4 DNA kinase (1 µg DNA in 10 µl reaction). Then these five pairs of oligos were annealed in separate reactions as described above. Five annealed oligo pairs were combined and ligated randomly into different promoter element multimer sequences in one reaction. Average size of ligated products was ~200 bps. DNA from the ligation reaction was gel purified to remove small DNA fragments (~100 bps and below) and unligated molecules. The ends of the purified DNA fragments were repaired by Klenow enzyme and cloned into expression vectors.

EXAMPLE 3: Cloning and Transient Assays of Synthetic Promoter Elements

To clone the synthetic promoters into expression vectors, AdhI intron-plus plasmid P1 (LexA::AdhI-89-minimal::Adh intron::LUC::PinII), and AdhI intron-minus plasmid P2 (LexA::AdhI-89-minimal::LUC::PinII) were digested with restriction enzymes to remove the LexA promoter element sequences. The cleaved sites were filled by Klenow enzyme, and the resulting backbone vectors were gel purified. The

synthetic promoters were ligated into these backbone expression vectors in separate reactions. About 20 positive clones for each construct were sequenced. Each ligated sequence was compared to the original promoter element sequences using either software Sequencher (GeneCodes, Ann Arbor, MI) or GAP (Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Based on the sequence information, seven constructs derived from AdhI intron-plus plasmids (Figure 4) and ten constructs derived from AdhI intron-minus plasmids were chosen for transient expression analysis (Figure 5).

Three-day-old seedlings of maize were bombarded with 3 µg of the experimental plasmids comprising SMPER::AdhI-89-minimal::Adh intron::LUC::PinII or those comprising SMPER::AdhI-89-minimal::LUC::PinII. (*See* Tomes, D. *et al.*, IN: Plant Cell, Tissue and Organ Culture: Fundamental Methods, Eds. O.L. Gamborg and G.C. Phillips, Chapter 8, pgs. 197-213 (1995), for general description of bombardment process.) Following 20 hours of incubation in the dark, crude protein extracts were prepared from roots and shoots. 20 µl tissue extracts were used for luciferase activity assays. For measurement of promoter activity, luciferase activity was used directly for each construct (Figure 6). The negative controls (plasmids P1 and P2) and their derivatives without the LexA sequence showed very low activity. The transient assays indicated that synthetic promoters comprising certain SMPERs can promote gene expression in maize. Therefore, only unique combinations of promoter elements generate functional promoters. Some synthetic promoters comprising particular SMPERs (A15, A18, A23, A24, A42, A44, A48, and A51 (Figure 6; sequences in Figures 7-14 (SEQ ID NO: 65-72)) exhibited enhanced gene expression.

EXAMPLE 4: Transformation and Regeneration of Transgenic Maize:

Biolistics:

The inventive polynucleotides contained within a vector are transformed into embryogenic maize callus by particle bombardment, generally as described by Tomes, D. *et al.*, IN: Plant Cell, Tissue and Organ Culture: Fundamental Methods, Eds. O.L. Gamborg and G.C. Phillips, Chapter 8, pgs. 197-213 (1995) and as briefly outlined below. Transgenic maize plants are produced by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids. The plasmids comprise a selectable marker gene and a structural gene of interest.

Preparation of Particles:

Fifteen mg of tungsten particles (General Electric), 0.5 to 1.8 μ, preferably 1 to 1.8 μ, and most preferably 1 μ, are added to 2 ml of concentrated nitric acid. This suspension was sonicated at 0°C for 20 minutes (Branson Sonifier Model 450, 40% output, constant duty cycle). Tungsten particles are pelleted by centrifugation at 10000 rpm (Biofuge) for one minute, and the supernatant is removed. Two milliliters of sterile distilled water are added to the pellet, and brief sonication is used to resuspend the particles. The suspension is pelleted, one milliliter of absolute ethanol is added to the pellet, and brief sonication is used to resuspend the particles. Rinsing, pelleting, and resuspending of the particles is performed two more times with sterile distilled water, and finally the particles are resuspended in two milliliters of sterile distilled water. The particles are subdivided into 250-ml aliquots and stored frozen.

Preparation of Particle-Plasmid DNA Association:

The stock of tungsten particles are sonicated briefly in a water bath sonicator (Branson Sonifier Model 450, 20% output, constant duty cycle) and 50 ml is transferred to a microfuge tube. All the vectors were cis: that is the selectable marker and the gene of interest were on the same plasmid. These vectors were then transformed either singly or in combination.

Plasmid DNA was added to the particles for a final DNA amount of 0.1 to 10 μg in 10 μL total volume, and briefly sonicated. Preferably, 10 μg (1 μg/μL in TE buffer) total DNA is used to mix DNA and particles for bombardment. Fifty microliters (50 μL) of sterile aqueous 2.5 M $CaCl_2$ are added, and the mixture is briefly sonicated and vortexed. Twenty microliters (20 μL) of sterile aqueous 0.1 M spermidine are added and the mixture is briefly sonicated and vortexed. The mixture is incubated at room temperature for 20 minutes with intermittent brief sonication. The particle suspension is centrifuged, and the supernatant is removed. Two hundred fifty microliters (250 μL) of absolute ethanol are added to the pellet, followed by brief sonication. The suspension is pelleted, the supernatant is removed, and 60 ml of absolute ethanol are added. The suspension is sonicated briefly before loading the particle-DNA agglomeration onto macrocarriers.

Preparation of Tissue:

Immature embryos of maize variety High Type II are the target for particle bombardment-mediated transformation. This genotype is the F_1 of two purebred

genetic lines, parents A and B, derived from the cross of two know maize inbreds, A188 and B73. Both parents are selected for high competence of somatic embryogenesis, according to Armstrong *et al.*, Maize Genetics Coop. News 65:92 (1991).

Ears from F_1 plants are selfed or sibbed, and embryos are aseptically dissected from developing caryopses when the scutellum first became opaque. This stage occurs about 9-13 days post-pollination, and most generally about 10 days post-pollination, depending on growth conditions. The embryos are about 0.75 to 1.5 millimeters long. Ears are surface sterilized with 20-50% Clorox for 30 minutes, followed by three rinses with sterile distilled water.

Immature embryos are cultured with the scutellum oriented upward, on embryogenic induction medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 2.88 gm/l L-proline, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, and 8.5 mg/l $AgNO_3$. Chu *et al.*, Sci. Sin. 18:659 (1975); Eriksson, Physiol. Plant 18:976 (1965). The medium is sterilized by autoclaving at 121°C for 15 minutes and dispensed into 100 X 25 mm Petri dishes. $AgNO_3$ is filter-sterilized and added to the medium after autoclaving. The tissues are cultured in complete darkness at 28°C. After about 3 to 7 days, most usually about 4 days, the scutellum of the embryo swells to about double its original size and the protuberances at the coleorhizal surface of the scutellum indicate the inception of embryogenic tissue. Up to 100% of the embryos display this response, but most commonly, the embryogenic response frequency is about 80%.

When the embryogenic response is observed, the embryos are transferred to a medium comprised of induction medium modified to contain 120 gm/l sucrose. The embryos are oriented with the coleorhizal pole, the embryogenically responsive tissue, upwards from the culture medium. Ten embryos per Petri dish are located in the center of a Petri dish in an area about 2 cm in diameter. The embryos are maintained on this medium for 3-16 hours, preferably 4 hours, in complete darkness at 28°C just prior to bombardment with particles associated with plasmid DNAs containing the selectable marker gene and structural gene or genes of interest.

To effect particle bombardment of embryos, the particle-DNA agglomerates are accelerated using a DuPont PDS-1000 particle acceleration device. The particle-DNA agglomeration is briefly sonicated and 10 ml were deposited on macrocarriers and the ethanol is allowed to evaporate. The macrocarrier is accelerated onto a stainless-steel stopping screen by the rupture of a polymer diaphragm (rupture disk). Rupture is effected by pressurized helium. The velocity of particle-DNA acceleration is determined

based on the rupture disk breaking pressure. Rupture disk pressures of 200 to 1800 psi are used, with 650 to 1100 psi being preferred, and about 900 psi being most highly preferred. Multiple disks are used to effect a range of rupture pressures.

The shelf containing the plate with embryos is placed 5.1 cm below the bottom of the macrocarrier platform (shelf #3). To effect particle bombardment of cultured immature embryos, a rupture disk and a macrocarrier with dried particle-DNA agglomerates are installed in the device. The He pressure delivered to the device is adjusted to 200 psi above the rupture disk breaking pressure. A Petri dish with the target embryos is placed into the vacuum chamber and located in the projected path of accelerated particles. A vacuum is created in the chamber, preferably about 28 in Hg. After operation of the device, the vacuum is released and the Petri dish is removed.

Bombarded embryos remain on the osmotically-adjusted medium during bombardment, and 1 to 4 days subsequently. The embryos are transferred to selection medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/1 thiamine HCl, 30 gm/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, 0.85 mg/l Ag NO_3 and 3 mg/l bialaphos (Herbiace, Meiji). Bialaphos is added filter-sterilized. The embryos are subcultured to fresh selection medium at 10 to 14 day intervals. After about 7 weeks, embryogenic tissue, putatively transformed for both the selectable marker gene and a structural gene or genes of interest, proliferates from about 7% of the bombarded embryos. Putative transgenic tissue is rescued, and that tissue derived from individual embryos is considered to be an event and is propagated independently on selection medium. Two cycles of clonal propagation are achieved by visual selection for the smallest contiguous fragments of organized embryogenic tissue.

A sample of tissue from each event is processed to recover DNA. The DNA is restricted with a restriction endonuclease and probed with primer sequences designed to amplify DNA sequences overlapping at least a portion of a synthetic multimeric promoter element region. Embryogenic tissue with amplifiable sequence is advanced to plant regeneration.

For regeneration of transgenic plants, embryogenic tissue is subcultured to a medium comprising MS salts and vitamins (Murashige & Skoog, Physiol. Plant 15: 473 (1962)), 100 mg/l myo-inositol, 60 gm/l sucrose, 3 gm/l Gelrite, 0.5 mg/l zeatin, 1 mg/l indole-3-acetic acid, 26.4 ng/l cis-trans-abscissic acid, and 3 mg/l bialaphos in 100 X 25 mm Petri dishes, and is incubated in darkness at 28°C until the development of well-formed, matured somatic embryos can be seen. This requires about 14 days. Well-formed somatic embryos are opaque and cream-colored, and are comprised of an

identifiable scutellum and coleoptile. The embryos are individually subcultured to a germination medium comprising MS salts and vitamins, 100 mg/l myo-inositol, 40 gm/l sucrose and 1.5 gm/l Gelrite in 100 X 25 mm Petri dishes and incubated under a 16 hour light:8 hour dark photoperiod and 40 meinsteinsm^{-2}sec^{-1} from cool-white fluorescent tubes. After about 7 days, the somatic embryos have germinated and have produced a well-defined shoot and root. The individual plants are subcultured to germination medium in 125 X 25 mm glass tubes to allow further plant development. The plants are maintained under a 16 hour light:8 hour dark photoperiod and 40 meinsteinsm^{-2}sec^{-1} from cool-white fluorescent tubes. After about 7 days, the plants are well-established and are transplanted to horticultural soil, hardened off, and potted into commercial greenhouse soil mixture and grown to sexual maturity in a greenhouse. An elite inbred line is used as a male to pollinate regenerated transgenic plants.

Agrobacterium-mediated transformation:

As a preferred alternative to particle bombardment, plants are transformed using Agrobacterium-mediated transformation. To construct transgenic vectors for this transformation, the synthetic promoters contained in the transient assay vectors (Example 3) were transferred to transgenic vectors by appropriate restriction digestion and ligation. The promoter fragments isolated from AdhI intron-plus transient vector P1 derivatives and from AdhI intron-minus transient vector P2 derivatives were ligated into the backbone of the transgenic vector P3 (GUS::PinII/2XCaMV35S::O'::AdhI intron::BAR::PinII) upstream of the GUS reporter sequence. The backbone of vector P3 was prepared by digestion of vector P3 to remove ubiquitin (UBI) promoter, 5' UTR, and UBI intron.

These resulting intermediate transgenic vectors were introduced into Agrobacterium tumefaciens LBA4404 by triparental matings to generate 'super-binary' vectors. Agrobaterium tumefaciens LBA4404 harboring the super-binary vector is used to transform maize.

For Agrobacterium-mediated transformation, the method of Zhao is employed (PCT patent publication WO98/32326, the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of Agrobacterium (step 1: the infection step). In this step the immature embryos are preferably immersed in an Agrobacterium suspension for the initiation of inoculation. The embryos are co-cultured for a time with the Agrobacterium (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation

period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of Agrobacterium without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of Agrobacterium and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step) and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants. Regenerated plants are monitored and scored for the activity of the gene of interest.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.